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Members of the basic-helix-loop-helix (bHLH) family of transcription factors are involved in various aspects of cell growth and differentiation, and the bHLH protein c-Myc has been implicated in breast cancer. We have determined that certain bHLH DNA binding region amino acids, which are critical for protein function but not required for high-affinity DNA binding, actually influence DNA recognition and binding conformation, and are required to maintain binding specificity. The data identify seemingly subtle effects on DNA binding that have important functional consequences, and they provide insights into how different bHLH proteins can act on different targets.

The zinc finger protein TTP is induced by many different growth factors, including EGF, and is required for regulation of TNFα activity. We have shown that forced TTP expression drives cells to proliferate, then undergo apoptosis, and can synergize with TNFα in promoting apoptosis. Our data suggest that TTP is important in growth factor signaling, and that its role in TNFα function is likely to be complex. Efforts that are underway to identify factors that interact with TTP-related proteins are likely to allow molecular investigations of how these proteins function, and to reveal new regulatory circuits that operate during growth factor responses.

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FOREWORD

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T. Keith Blackwell 11/24/97

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INTRODUCTION:

Foreword:

Since this project was originally funded, its aims have been modified so that they consist of 1) mutagenesis studies of dimerization and DNA binding by c-Myc and other bHLH proteins, 2) investigation of whether the Mastermind protein, an important component of the Notch signaling pathway, functions as a sequence-specific DNA binding protein, and 3) investigation of the functions of TTP/TIS11 proteins. These modified aims have been approved by Dr. Patricia Modrow at the USAMRMC. As indicated in last year's report, we have encountered considerable technical difficulties in the dimerization experiments outlined in aim 1. None of the mutants we have made has yielded experimental data that can be interpreted in a straightforward manner, suggesting that bHLH dimerization affinities are determined by multiple complex factors. In that aim, therefore, we have focused entirely on the DNA binding experiments, which have made progress as outlined below. Also, although our initial efforts at identification of specific Mastermind binding sites yielded encouraging results, during the past year we have been able to demonstrate only non-specific binding, and have not detected synergy with the candidate co-factor protein Su (H). We interpret these findings to indicate that Mastermind is likely to require an as-yet-unidentified co-factor to bind DNA. Given that identification of such a co-factor would require a time-consuming fishing expedition, we have instead directed our efforts toward the TTP/TIS11 project, which is moving forward.

As both the dimerization and Mastermind experiments have been described in previous reports, here I will discuss the DNA binding and TTP/TIS11 projects, on which we are making progress. Each section will be divided into two parts, each of which will correspond to one of these projects. Given our progress so far, we expect to submit a manuscript in each of these areas within the coming months.

Determinants of bHLH protein DNA binding specificity:

The basic-helix-loop-helix (bHLH) motif (Figure 1) (Murre et al. 1989a; Murre et al. 1989b) defines a large family of transcriptional regulators. Some bHLH proteins mediate cellular differentiation, and others are involved in cell proliferation and/or have been implicated in oncogenesis (Weintraub et al. 1991a). These proteins form dimers through the HLH segment, which lies immediately C-terminal to the basic region (BR), through which they bind DNA (Davis et al. 1990; Murre et al. 1989b; Voronova and Baltimore 1990). Myc proteins belong to a bHLH subgroup (bHLH-ZIP proteins) in which a "leucine zipper" (ZIP) segment is located immediately C-terminal to the HLH segment (Blackwood and Eisenman 1991), and provides a critical contribution to dimerization (Beckmann and Kadesch 1991; Davis and Halazonetis 1993; Ferre-D' Amare et al. 1993; Fisher et al. 1991; Halazonetis and Kandil 1992; Ma et al. 1993). bHLH proteins bind DNA as dimers (Davis et al. 1990; Voronova and Baltimore 1990), and generally recognize sites that contain the palindromic consensus CA -- TG (Lassar et al. 1989), with each respective BR binding to half of the site (Blackwell and Weintraub 1990; Ferre-D' Amare et al. 1993). Some bHLH protein family members readily form homodimers, but others do not, and

appear to require a different dimerization partner (Weintraub et al. 1991a). For example, while Myc protein bHLH-ZIP domains can bind DNA *in vitro* as homodimers (Alex et al. 1992; Blackwell et al. 1990; Kerkhoff et al. 1991; Ma et al. 1993), they dimerize (and thus bind DNA) far more efficiently as heterodimers with Max, a widely-expressed bHLH-ZIP protein (Blackwood and Eisenman 1991; Prendergast et al. 1991) which appears to be essential for their normal functions (see (Amati et al. 1993)). Similarly, MyoD, and other closely-related bHLH proteins involved in myogenesis, form homodimers relatively poorly, but form heterodimers and bind DNA well with the ubiquitously-expressed 'E' group of bHLH proteins (Chakraborty et al. 1991; Davis et al. 1990). These "myogenic" bHLH proteins apparently mediate myogenic differentiation as heterodimers with E proteins (Lassar et al. 1991; Neuhold and Wold 1993).

Crystallographic analysis of Max homodimers has shown that the HLH segment consists of a parallel, left-handed, four helix bundle, from which the ZIP domain continues C-terminally, and the BR extends as an α-helix N-terminally from helix 1 to cross the major groove (Figure 1) (Ferre-D' Amare et al. 1993). Structures of homodimers of the bHLH proteins E47 (an E protein) and MyoD (which lack a ZIP domain) bound to DNA have further revealed that the configuration of the HLH domain fold is remarkably preserved between bHLH and bHLH-ZIP proteins (Ellenberger et al. 1994; Ma et al. 1994a). These structures have demonstrated how the HLH dimerization interface is formed, and have made predictions about critical protein-DNA contacts, but also leave open a number of questions. For example, they have not suggested roles for a number of BR residues which do not contact bases, yet are conserved within different bHLH protein sub-families (Benezra et al. 1990), and thus might be essential for their function. They also do not reveal how bHLH protein DNA binding preferences within and around the CA -- TG consensus (Blackwell et al. 1990; Blackwell and Weintraub 1990) are determined, because particular important bases are not directly contacted in these structures. These issues are important, because different members of the bHLH protein family act on different sets of genes, and have widely divergent biological functions, yet bind to apparently similar CANNTG sites (Weintraub et al. 1991a).

Myc-family bHLH proteins bind to sites with CACGTG or CATGTG cores (Alex et al. 1992; Berberich et al. 1992; Blackwell et al. 1990; Kato et al. 1992; Ma et al. 1993; Papoulas et al. 1992), suggesting similar or overlapping functions. However, a number of related bHLH proteins, including the bHLH-ZIP transcriptional regulatory proteins USF, TFE3, and TFEB, can also bind to the same sequences (Beckmann et al. 1990; Carr and Sharp 1990; Gregor et al. 1990). All of these bHLH-ZIP proteins contain in their respective BRs an arginine (R) residue (R_{13}, Figure 1) which is essential for recognition of these particular CA -- TG sites (Blackwell et al. 1993; Dang et al. 1992; Halazonetis and Kandil 1992; Van Antwerp et al. 1992), and which directly contacts the central bases (Ferre-D' Amare et al. 1993). These similarities in DNA recognition raise the issue of how Myc proteins and these other bHLH-ZIP proteins might be able to act on different genes, and would appear to suggest that any differences in their target specificities would be determined by interactions with cooperating factors. Conversely, some differences in DNA recognition have been identified among these proteins (Blackwell et al. 1993; Fisher and Goding 1992; Halazonetis and Kandil 1991; Prochownik and Van Antwerp 1993).

For example, the ability to bind to certain "non-canonical" sites, which are based on variants of the CA -- TG consensus, is shared by the Myc-family proteins, but not by the other related bHLH-ZIP proteins, indicating that it might confer some degree of specificity and thus be of biological significance (Blackwell et al. 1993). Such DNA sequences are associated with a number of candidate Myc-responsive genes (Grandori et al. 1996).

The example of MyoD (Figure 1) suggests that the relationship between DNA-binding and transcriptional regulation by c-Myc might be complex. Mutational analyses of MyoD, and of related bHLH proteins, have shown that particular BR mutations allow binding to appropriate DNA sequences, but interfere with activation of transcription and/or myogenesis (Davis et al. 1990; Davis and Weintraub 1992; Schwarz et al. 1992; Weintraub et al. 1991b). These findings suggest that the MyoD BR is involved in protein-protein interactions, as well as in DNA binding (Weintraub et al. 1991b). Certain of these mutations behave differently in different cell lines (Weintraub et al. 1991b), further supporting the idea that other co-factor(s) interact with the DNA-bound BR. This mechanism could potentially contribute to target specificity, if only a subset of MyoD binding sites were to allow binding in a conformation which would permit these protein-protein interactions to occur (Weintraub et al. 1991b), or if particular promoter contexts were required for an appropriate complement of co-factors to be present (Weintraub et al. 1994). In these experiments, three MyoD residues (BR positions 5, 6, and 15; Figures 1 and 3) were identified as essential for myogenesis, but not DNA binding (Davis and Weintraub 1992). I will refer to these MyoD residues as "myogenic" residues. As is explained below, this model is complicated by the finding that residues 5 and 6 are oriented toward the DNA (Ma et al. 1994a), indicating that any influence they might have on interactions with other proteins must be indirect.

Such complex mechanisms for determining target specificity could potentially be utilized by other bHLH proteins, including those of the Myc family. In fact, members of different bHLH protein subgroups, including the Myc proteins, are characterized by conserved BR amino acids that do not seem to be involved in DNA-binding specificity (Ferre-D' Amare et al. 1993; Fisher et al. 1993). In the original submission of this grant, I proposed to conduct a thorough functional analysis of the c-Myc BR residues that correspond to the critical MyoD BR amino acids mentioned above. As I was beginning these studies, I became aware that another laboratory had determined that one of these, c-Myc BR residue 6 (Figure 1), was not required for either transformation or apoptosis (Bodis et al. 1997). This observation appears to suggest that these BR residues are not essential for c-Myc function *in vivo*, although it remains possible that the assays employed do not completely reflect all of the biological functions of c-Myc. In light of this finding, I chose to concentrate on investigating the role of these residues in MyoD, in which their biological effects have been defined by extensive mutagenesis studies.

As is described in the next section, we have obtained evidence that although these residues are not essential for high affinity binding of MyoD to its cognate site, they do influence its DNA binding specificity. Particular mutations that abolish myogenic activity result in MyoD binding to DNA with the specificity characteristic of the bHLH protein Twist, which *in vivo* is required for mesoderm formation (Michelson 1996), but may inhibit MyoD function (Huang et al. 1996; Spicer et al.

1996). Twist and the resulting MyoD chimeras bind preferentially to a CATATG site, but with less specificity than wild-type MyoD binds to its preferred recognition sequence. Mutations of individual residues can determine preferences over the entire binding site, suggesting that these residues influence the conformation of the DNA-bound BR. The results suggest that bHLH proteins can recognize DNA through binding in a finite number of different conformations, and they predict that the specificity of putative co-factors that recognize the bound BR would be determined by these conformations. They are also consistent with the idea that subtle differences in binding specificity alone could be critical for restricting bHLH protein activity.

TTP/TIS11 proteins, cell proliferation, and TNFα activity:

TTP/TIS11/Nup475 (referred to as TTP) is an immediate-early gene that is induced transiently by serum treatment of cultured mammalian cells (DuBois et al. 1990; Lai et al. 1990; Varnum et al. 1991). Among the stimuli that induce TTP, or the related genes TIS11b and TIS11d, are insulin, TGFα, PDGF, FGF, TPA, antibody-capping of B lymphocytes, and epidermal growth factor (EGF), which has been implicated in breast cancer (Bustin et al. 1994; Corps and Brown 1995; Gomperts et al. 1992; Mittelstadt and DeFranco 1993; Wang et al. 1994). The TTP gene is also induced *in vivo* during regeneration of certain types of tissue, such as liver and intestine (DuBois et al. 1990; Ehrenfried et al. 1995)

Of the three mammalian TTP/TIS11 genes, only TTP has been disrupted in mice. The TTP-/- mice have a syndrome of polyarthritis, autoimmunity, cachexia, alopecia, dermatitis, lymphadenopathy, and myeloid hyperplasia, a constellation of symptoms which is very similar to that resulting from transgenic expression of tumor necrosis factor-α (TNFα) (Taylor et al. 1996a). Remarkably, the TTP-deficient mice are "cured" by regular injections of anti-TNFα antibodies (Taylor et al. 1996a). This phenotype can be transfered by bone marrow transplantation, and macrophages from these mice over-produce TNFα in response to LPS (Carballo et al. 1997). The simplest interpretation of these findings is that TTP is involved directly in regulation of TNFα production in lymphoid cells. However, given evidence that these proteins play a broader role (see below), it is also plausible that this phenotype could arise indirectly, from an effect on lymphoid cell proliferation or signaling, or auto-feedback responses to TNFα. An understanding of how TTP/TIS11 proteins function at the molecular level will therefore illuminate a genetically-identified key step in TNFα action, and provide insights into molecular regulatory circuits that are involved in the responses to multiple different growth factors, including EGF.

TTP/TIS11 proteins are defined by two tandem zinc fingers of the Cys-Cys-Cys-His (CCCH) class (Figure 2), a type about which relatively little is known (Berg and Shi 1996). Humans and rodents have three TTP/TIS11-related genes (TTP, TIS11b and TIS11d), which are very similar within these zinc fingers (Figure 2; not shown), and in size, but are otherwise divergent. TTP and TIS11b/d orthologs have also been identified in Drosophila, Xenopus, C. elegans, and yeast (Ma and Herschman 1995; Ma et al. 1994b) (T. K. Blackwell, data not shown). Genetic and molecular studies have implicated other proteins that contain CCCH zinc fingers in mRNA processing, or in gene regulation at the transcriptional or post-

transcriptional levels (Barabino et al. 1997; Guedes and Priess 1997; Mello et al. 1996; Seydoux et al. 1996; Zhang 1992). For example, the C. elegans PIE-1 protein (Figure 2) appears to encode a broadly-acting repressor of mRNA transcription, but the role played by its CCCH zinc fingers is unknown (Mello et al. 1996; Seydoux and Dunn 1997; Seydoux et al. 1996), (C. Batchelder and T. K. Blackwell, unpublished). Some CCCH zinc finger proteins bind RNA, or are associated with RNA-binding proteins (Bai and Tolias 1996; Barabino et al. 1997; Murray et al. 1997; Zhang 1992). However, despite this association with RNA binding, it has not been determined at the molecular level exactly how any CCCH zinc fingers function.

In cultured mammalian fibroblasts, TTP is detected in the nucleus (DuBois et al. 1990), but may translocate to the cytoplasm when its expression is induced during the earliest phases of growth factor responses (Taylor et al. 1996b). TIS11b and d may be expressed more constitutively, but are also induced by multiple different growth factors (Corps and Brown 1995; Gomperts et al. 1992). In adult mice, TTP is expressed in organs that contain lymphoid tissue (Taylor et al. 1996a), but relatively little is known about the embryonic pattern or cell-type specificity of TTP/TIS11 gene expression. In Drosophila (Ma et al. 1994b) and Xenopus (see below), these genes are expressed in the earliest embryonic stages, suggesting broad and perhaps overlapping functions. In the fission yeast S. pombe, lack of a TTP/TIS11 ortholog prevents pheromone-induced sporulation and mating (Kanoh et al. 1995). This mutation can be partially overcome by constitutive expression of the appropriate MAP kinase, or an activated *ras* gene, but over-expression of the TTP/TIS11 gene cannot overcome defects at various points in the signaling cascade (Kanoh et al. 1995). These observations are important, because they indicate that the TTP/TIS11 protein is required for this signaling response to function or to be effected, but is not an integral component of the pathway (Kanoh et al. 1995). In S. cerevisiae, overexpression of a TIS11b/d-related protein can complement simultaneous temperature-sensitive mutations in the cell-cycle regulators *nim-1* and *cdc25*, suggesting that it can promote proliferation (Warbrick and Glover 1994). On the other hand, in a different study, overexpression of TTP (or of related yeast genes) appeared to retard growth of S. cerevisiae (Thompson et al. 1996). This complex picture is consistent with TTP/TIS11 proteins regulating expression of genes involved in responses to various diverse growth factors, perhaps including regulation of genes involved in cell cycle entry.

We have determined that overexpression of TTP in mammalian cell lines induces proliferation, followed by apoptotic cell death. This finding appears to be analogous to the observation that expression of c-Myc (Evan et al. 1992) or CDC25 (Galaktionov et al. 1996), which induce proliferation, can cause apoptosis if growth factors are not present. Lack of growth factors is not required for TTP to induce apoptosis, but does exacerbate this effect. Like c-Myc, TTP also acts synergistically with TNFα to induce apoptosis. The results suggest that TTP can promote cell-cycle entry or affect cell-cycle progression, consistent with its putative involvement in growth factor responses, and that its constitutive expression results in a regulatory perturbation that triggers programmed cell death. Efforts are underway or planned to examine further the effect of TTP expression on the cell cycle, to investigate the functions of TIS11b/d proteins, to identify factors that interact with these proteins, and to test directly whether TTP can regulate expression of TNFα.

BODY:

Determinants of bHLH protein DNA binding specificity:

As indicated in earlier reports, we have obtained evidence that the "myogenic" residues in the MyoD BR influence DNA binding specificity in a way that is most consistent with an effect on positioning and/or conformation of the BR. During the past year, we have obtained further evidence that multiple BR mutations that eliminate MyoD biological activity, but allow DNA binding, result in a "Twist-like" binding preference. Our experiments also show that Twist:E protein heterodimers, and these inactive MyoD mutants, bind DNA with specificities that are similarly relaxed with respect to recognition of CANNTG sites, and that a relaxed binding specificity correlates with a lack of biological activity. We have now begun to construct mutants to test specifically whether the myogenic residues *per se* mediate the observed effects on BR positioning.

To evaluate the influences of the myogenic residues on DNA binding, we first identified sequences preferentially bound by various MyoD mutants, using the selection and and amplification of binding sites (SAAB) technique of *in vitro* nucleic acid selection (Blackwell and Weintraub 1990). In these experiments, we created sequence libraries in which positions within and flanking the CANNTG bHLH consensus were randomized (Figure 4), then performed multiple reiterative rounds of selection for binding. Since the CANNTG consensus fixes the position of binding, the resulting selected sites can be sequenced as a pool (Blackwell et al. 1990; Blackwell and Weintraub 1990). This strategy provides an unbiased and very sensitive indicator of alterations in binding preference that are subtle, and might not be identified through conventional approaches

When the MyoD BR is replaced with that of E12 (an E2A protein, Figure 1), the resulting mutant protein (MD/E12basic; Figure 3) binds to a muscle-specific regulatory element as a heterodimer with E2A, but will not induce myogenesis or activate transcription through a complex muscle-specific enhancer (Davis et al. 1990; Davis and Weintraub 1992; Weintraub et al. 1991b). Similar results have been obtained when the E12 BR was substituted into the myogenic bHLH protein Myogenin (Brennan et al. 1991). To determine whether this mutation affects DNA binding preference, we performed a SAAB experiment on homodimers of MD/E12basic. While MyoD binds preferentially to the sequence G/AACAGCTGTT/C (Figure 5, (Blackwell and Weintraub 1990)), MD/E12basic prefers the sequence G/ACCATATGGT/C, which differs from the MyoD site over the 8 central base pairs (Figure 5). This site also differs significantly from the CACCTG sequence preferred by E2A proteins (Blackwell and Weintraub 1990), indicating that the binding preference of the E12 BR can vary depending upon its molecular context. Back-substitution of A_5 of MyoD into MD/E12basic does not allow myogenesis (Weintraub et al. 1991b), and results in preferences that are more MyoD-like at positions ± 4, but otherwise has little effect on binding (MD/E12basic-A; Figure 3). However, substitution of both A_5 and T_6, which restores the capability to induce myogenesis in cell culture assays (Brennan et al. 1991; Weintraub et al. 1991b), results in preferences that are indistinguishable from those of wild-type

MyoD (MD/E12basic-AT; Figure 3). The data indicate that substitution of these individual amino acid residues influences binding preferences over the entire range of each 5 bp half-site, and that the myogenic activity of these proteins correlates with their DNA binding preferences.

To investigate how heterodimer formation influences DNA binding by these proteins, we performed SAAB experiments on various MyoD and E12 mutant combinations (Figure 6). As reported previously (Blackwell and Weintraub 1990) MyoD+E12 heterodimers select from the D3 library a MyoD-like half site at positions +4 and +5, an E2A-like half-site at -4 and -4, and CC or GG bases in the center of the site (Figure 6), indicating asymmetric binding. Some of these preferences are subtle, because MyoD + E12 heterodimers bind with comparable affinity to a preferred MyoD homodimer site, and MyoD homodimers bind well to an asymmetric heterodimer sequence (Blackwell and Weintraub 1990). Heterodimers of MD/E12basic+E12 prefer wild-type heterodimer sequences in the center of the site, but select E2A-like sequences in both flanking regions, at ± 4 and ± 5 (Figure 6). Similarly, a heterodimer of MyoD and an E12 protein containing the MyoD BR (E12/MDbasic; Figure 3) also selects a wild-type heterodimer preference within the CANNTG motif, but binds with a MyoD-like preference at ± 4 and ± 5 (Figure 6). These patterns are distinct from the MD/E12basic homodimer preference, which differs from both the MD and E12 BR half site preferences throughout the site (Figure 5). Apparently, when only one BR is swapped, the binding preferences of these dimers correspond to those of the wild type BRs at positions outside the CANNTG consensus.

In contrast, MD/E12basic +E12/MDbasic heterodimers, in which both BRs are substituted, prefer sites that are more similar to those selected by MD/E12basic (Figure 5), including the AT sequence in the center of the site (Figure 6). This last observation indicates that binding preferences are affected over the entire site when both of these BRs are mis-paired relative to their protein contexts, a situation that is perhaps analogous to MD/E12basic homodimers. It suggests that BR positioning is important for determining binding sequence preferences.

The binding sites prefered by MD/E12basic and MD/E12B-A homodimers, and by MD/E12basic + E12/MDbasic heterodimers, are remarkably similar to the preferences of the bHLH protein Twist (Figure 6). Twist homodimers and Twist+E12 heterodimers both prefer the core sequence CATATG, which corresponds to natural Twist-responsive regulatory elements (Szymanski and Levine 1995). They also select MyoD-like sequences at ± 5, and are similar to MD/E12B-A in their preferences at ± 4 (Figure 6). The data are consistent with the notion that the A_5, N_6 sequence common to Twist and MD/E12B-A BRs (Figure 3) is involved in mediating the corresponding preferences observed at ± 4 and ± 5. However, it is surprising that the Twist core preferences are identical to those associated with mis-pairing of both MyoD and E12 BRs, because these proteins lack obvious BR similarities aside from the conserved residues (Figures 5 and 6).

Mutagenesis experiments have indicated that the BR-HLH junction region is critical for myogenesis. For example, a MyoD protein containing the E12 BR and junction regions (MD/E12BJ, Figure 3) does not induce myogenesis when introduced into cultured cells either alone, or in a co-transfection with E12 (Davis and Weintraub 1992). However, myogenesis is observed when MD/E12BJ is

transfected together with the corresponding E12 mutant containing the MyoD BR and junction regions (E12/MDBJ, Figure 3), but not when the reciprocal BR mutants (MD/E12basic + E12/MDbasic, Figure 3) are introduced together (Davis and Weintraub 1992). E12/MDbasic alone is also inactive in this assay (Figure 3), but E12/MDBJ, which contains only four additional MyoD residues (Figure 3) can induce myogenesis when transfected alone, presumably through pairing with endogenous E proteins (Davis and Weintraub 1992). In fact, an E12 protein containing only the three "myogenic" MyoD residues, A_5 and T_6 from the BR, and K_{15} from the junction region (E12/AT,K; Figure 3), can both transactivate a muscle-specific reporter and induce myogenesis in tissue culture (Davis and Weintraub 1992).

The results described in Figures 5 and 6 suggest that aspects of BR positioning, as reflected by binding sequence preferences, are critical for the myogenic function of MyoD. It would be predicted, then, that BR-HLH junction residues might influence these binding preferences. A heterodimer of MD/E12basic + E12/MDbasic binds to both MyoD and Twist preferred sites (Figure 7, lanes 4 and 10), as would be predicted from the SAAB pattern (Figure 6). In contrast, MD/E12BJ + E12/MDBJ heterodimers clearly prefer the MD site (Figure 7, lanes 5 and 11), as do MyoD + E12 heterodimers (Figure 7, lanes 1 and 7). This observation indicates that pairing of BR and junction residues influences DNA binding preferences. Significantly, our findings also indicate that preferences for MyoD vs. Twist sites generally correlates with the biological activity of these mutant proteins, so that the Twist preference is associated with a lack of myogenesis. Consistent with the observation that MD/E12BJ does not induce myogenesis when transfected alone, although a heterodimer of this protein with E12 prefers a MyoD-like site, its binding affnity is notably lower than that of MD/E12BJ + E12/MDBJ heterodimers (Figure 7, lanes 3, 5, 9, and 11).

Although Twist + E12 heterodimers appear to bind better to the Twist site than to the MyoD site (Figure 7, lanes 2 and 8), their preference for this selected sequence appears to be lower than that of MyoD + E12 heterodimers for a MyoD-like site (Figure 7, lanes 1 and 7). This observation raises an important issue, that of the extent to which the DNA binding preferences of these proteins reflect their binding specificities for particular CANNTG sequences. We have addressed this question using binding competition assays (Figures 8 and 9). Binding of MyoD and MyoD + E12 complexes to the MyoD site is competed much more effectively by the MyoD sequence than by Twist or c-Myc (CACGTG) preferred sites, both of which compete better than the Skn-1 binding site, which lacks a CANNTG motif (Figure 8 A-D, lanes 2, 5, 8, 11, 14, and 17). A similar competition pattern is seen for lower-affinity binding of MyoD + E12 homodimers to the Twist consensus (Figure 9 A-D, lanes 2, 5, 8, 11, 14, and 17). In contrast, although Twist + E12 complexes bind preferentially to the CATATG motif found in natural Twist-responsive elements (Szymanski and Levine 1995) (Figure 6), this binding is competed almost equally well by MyoD, Twist, and c-Myc binding sites (Figure 9 A-C, lanes 7, 13, and 19). Together with the lack of competition by the Skn-1 binding site (Figure 9 D, lanes 7, 13, and 19), this observation indicates that Twist + E12 complexes can specifically recognize a wide variety of CANNTG sequences. A similar pattern is characteristic of Twist homodimers (Figure 9 A-D, lanes 4, 10, and 16).

Heterodimers of MD/E12basic + E12 select sequences preferred by E2A proteins (Figure 6), which also bind well to sites preferred by MyoD (Blackwell and Weintraub 1990) (not shown). However, like Twist + E12 homodimers, these MD/E12basic + E12 complexes do not significantly discriminate between the preferred MyoD and Twist consensuses (Figures 8 and 9, A-D, lanes 6, 12, and 18). Thus, although these MD/E12basic + E12heterodimers do not select Twist sites in the SAAB assay (Figure 6), consistent with their lack of biological activity (Davis and Weintraub 1992), they also lack specificity for binding to MyoD- or E2A-like sites. MD/E12basic homodimers, in contrast, bind to the Twist consensus site with greater specificity than either of these heterodimer species bind to their respective preferred sites (Figure 9, A-C, lanes 3, 9, and 15; Figure 10B, lanes 2, 5, 8, 11, 14, and 17). This is consistent with the finding that MyoD/E12basic is also more selective in the SAAB assay (Figures 5 and 6). Also consistent with the SAAB results, MD/E12B-A and MD/E12B-AT bind specifically to the Twist and MyoD sites, respectively (Figure 10).

Our findings indicate that the A_5 and T_6 residues are essential for a MyoD-like binding preference, and that juxtaposition of BR and junction residues has a profound effect on DNA binding preferences. They have also shown that a MyoD- or E- like binding preference is associated with biological activity, provided that specificity for these DNA sequences is maintained. It appears likely, then, that the myogenic residues A_5, T_6, and K_{15} together establish the MyoD binding preference, and that this underlies their functional importance. To test this observation directly, we have begun constructing a series of mutants in which non-essential MyoD residues have been substituted with alanine (Fisher et al. 1993), and residues at BR/junction positions 5, 6, and 15 have been substituted with those found in E2A and Twist proteins (Figure 11). These mutants have been designed so that BR/junction combinations among them can be readily constructed. These combinations are being produced currently. We will test these mutants in the competition assay describe in Figures 8-10 and, if necessary, in the SAAB assay. These experiments should determine whether, in these proteins, these residues alone specify binding preferences for different CANNTG sites.

TTP/TIS11 proteins, cell proliferation, and TNFα activity:

As indicated above, the similarities between the TTP/TIS11 proteins and the C. elegans PIE-1 protein (Figure 2) suggested that TTP/TIS11 proteins might also function as transcriptional repressors. When linked to the yeast GAL4 DNA binding domain, multiple regions of PIE-1 repress promoters to which they are tethered nearby through GAL4 binding sites (C. Batchelder and T. K. Blackwell, unpublished). A particularly powerful repressor domain C-terminal to the PIE-1 zinc fingers has been mapped in detail, and analyzed by mutagenesis (C. Batchelder and T. K. Blackwell, unpublished). Although TIS11/TTP proteins lack sequences that closely resemble this repressor domain, it remains possible that they contain other types of repressor regions. To test this idea, we separately linked the TTP residues N-terminal to the zinc fingers, the zinc finger region, and the residues C-terminal to the TTP zinc fingers, to the GAL4 DNA binding domain. Of these constructs, only the zinc finger region had any repressor activity (not shown). We have detected such activity in other CCCH zinc fingers, including some from

proteins that are predominantly cytoplasmic (not shown). Although we are not sure of the significance of this finding, it may indicate that the PIE-1 and TTP zinc fingers act on similar targets. These observations do not support, but also do not rule out, the idea that TTP might function as a transcription factor.

In light of the evidence that TTP moves from the nucleus to the cytoplasm when its expression is induced (Taylor et al. 1996b), the links between CCCH zinc fingers and RNA binding (see above), and genetic evidence that some CCCH proteins function post-transcriptionally (Guedes and Priess 1997), it seems reasonable that TIS11/TTP proteins might act at the post transcriptional level. We have begun to investigate TTP function by determining the effects of its expression in the cell, and by looking for factors that interact with TTP.

The apparent role of TIS11/TTP proteins in growth factor/mitogen/cytokine responses suggests that expression of these proteins might have an effect on cell cycle entry or progression, and that analysis of this effect could identify regulatory circuits on which they normally act. To test the effects of TTP expression in cultured cells, we have subcloned the murine TTP cDNA into a CMV-based mammalian cell expression vector. By introducing a β-galactosidase expression vector into cells together with this construct, it is possible to identify co-transfected cells that express TTP by staining for β-galactosidase activity. Within 24 hours after transfection, murine 3T3 fibroblast cells that express β-galactosidase, and presumably TTP, are more numerous than controls, suggesting that TTP expression induces these cells to proliferate (Figures 12A, 12B, and 13). However, an increased proportion of the TTP-expressing cells are undergoing apoptosis (Figures 12A, 12B, and 13). In these experiments, apoptosis is measured as the number of obviously dead cells still adherent to the plate, and has been confirmed by co-transfection of TTP with a GFP-expression vector, combined with Hoechst staining, to identify transfected nuclei with a characteristic apoptotic appearance (not shown). These assays probably underestimate the extent of apoptosis, because they do not count cells that are no longer adherent. However, by 48 hours post-transfection, the majority of TTP-expressing cells are dead or appear to be in the process of dying (Figures 12C, 12D, and 13). Similar results have been obtained using HeLa cells (not shown).

3T3 cells that have been deprived of serum do not proliferate in response to TTP expression but, instead, undergo extensive apoptosis by both 24 and 48 hrs. post-transfection (not shown). These findings are consistent with the idea that TTP induces cell death by directing cells to enter or progress through the cell cycle inappropriately. They suggest that constitutive TTP expression may potentiate some aspect of a proliferative signal, and that cell death then occurs because of abnormal timing or intensity of that signal. This effect is exacerbated if the cells are arrested because of a lack of growth factors. In support of this model, preliminary FACS analyses of these TTP-transfected cells indicate that an increased proportion are in the G2 phase of the cell cycle (not shown), indicating perhaps that the cells are progressing more rapidly through other cell cycle phases, and also that they may be attempting a G2 growth arrest.

Treatment of cells with TNFα activates multiple signaling pathways, including an apoptotic stimulus, the NF-κB transcription factor, and the Jun N-terminal kinase (Chinnaiyan et al. 1995; Hsu et al. 1996; Liu et al. 1996). In most cell lines, TNFα treatment induces cell death only if protein synthesis is inhibited by

cycloheximide, because the NF-κB activity that is induced simultaneously blocks the apoptotic signal (Beg and Baltimore 1996; Liu et al. 1996; Van Antwerp et al. 1996; Wang et al. 1996). However, forced expression of c-Myc allows TNFα to induce apoptosis in the absence of cycloheximide, and this effect appears to involve G1 cyclins (Janicke et al. 1996). The extent to which these pathways are intertwined is illustrated by the observation that certain cyclin-dependent kinases inhibit NF-κB function by binding to p300, a co-factor that appears to be required for NF-κB to activate downstream genes (Perkins et al. 1997). Expression of c-Myc and TNFα treatment thus might synergistically induce cell death through down-regulation of NF-κB by the growth stimulus from c-Myc, and/or through growth inhibition by TNFα causing c-Myc to trigger apoptosis.

We have determined that forced TTP expression also synergizes with TNFα treatment to induce programmed cell death. TNFα appears to inhibit cell proliferation, but at 24 hr. post-transfection it triggers considerable apoptosis in TTP-expressing cells, in the absence of cycloheximide (compare Figures 13 and 14). At 48 hr. post transfection, the proportion of apoptotic cells appears comparable in TNFα-treated and untreated cells (compare Figures 13 and 14). However, the extent of cell death is underestimated at both time points in the population of TNFα-treated cells that express TTP, because numerous dead cells are no longer adherent to the plate (not shown). Our findings suggest that TTP and c-Myc might similarly synergize with TNFα in promoting apoptosis. They are also consistent with the possibilities that TTP might inhibit NF-κB -induced genes that block the apoptotic signal, that growth inhibition by TNFα might contribute to TTP-driven apoptosis, and that TTP might be involved in modulating cellular responses to TNFα.

To investigate directly how TIS11/TTP proteins function in the cell, we have begun efforts to identify protein factors that interact with them. We are setting up a two-hybrid screen using TTP as the bait, and have raised an antibody against a peptide from the TTP amino terminus . This antibody works extremely well in western blotting assays (not shown). We will use this antibody for analyses of TTP interactions with proteins derived from the two hybrid screen, for immunoprecipitation of TTP-containing complexes from cell lysates, and for immunocytochemistry. We are also in the process of expressing a GST-TTP fusion protein for use in the interaction assays, and of expressing TIS11b and TIS11d in cultured cells to investigate whether these proteins function similarly to TTP. Finally, we have cloned two TIS11b- and TIS11d-related genes from Xenopus (not shown), and plan to investigate by *in situ* hybridization when those genes, and the mouse TIS11/TTP genes, are expressed in the developing embryo.

CONCLUSIONS:

Our experiments indicate that the "myogenic" residues in the MyoD BR, A_5 and T_6, have a marked effect on DNA binding preference that correlates with biological activity. In the context of the MD/E12basic mutant, a "MyoD-like" binding preference is associated with proteins that are capable of inducing myogenesis, and a "Twist-like" preference is associated with a lack of activity. Mutations at these residues are associated with preference changes over a 5 bp half-

site, indicating that these changes must involve differences in BR conformation or positioning. X-ray crystallographic analysis has revealed that the A_5 and T_6 residues (Figures 1 and 3) allow MyoD to pack more tightly into the major groove than do the corresponding N_5 and N_6 residues of E2A proteins (Ma et al. 1994a). This allows R_2 (Figures 1 and 3) to be buried in the DNA, and contact a base at ± 3 within the CANNTG consensus (Ma et al. 1994a). It also allows R_1 to contact a backbone phosphate at ± 6 (Ma et al. 1994a). In contrast, in E2A and Max proteins, R_2 adopts a different conformation and contacts the backbone, and R_1 does not interact with the corresponding DNA (Ellenberger et al. 1994; Ferre-D' Amare et al. 1993). It appears plausible, then, that the particular conformations of these arginine residues mediate the distinctive preference of MyoD at positions ± 4 and ± 5. However, it is not clear from these structures why mutations at BR positions 5 and 6 would influence binding preferences over the entire site.

With regard to BR positioning, it is significant that proper pairing of BR and junction residues appears to be necessary for either MyoD or E2A-like binding preferences, and that mis-pairing can result in a Twist-like binding preference (Figures 5-7). This observation suggests that residues at BR positions 5 and 6 act in concert with the junction region to position the BR in the major groove. Again, in these experiments, proteins that are inactive in the myogenesis assay either prefer a Twist site, or lack specificity for a MyoD site. This link between binding preference and biological function, together with the observation that junction residue K_{15} is involved in myogenesis, suggests that this residue might also mediate the effect of the junction on DNA binding. This residue is oriented away from the DNA and the dimerization interface (Ma et al. 1994a), but could potentially have a conformational effect by influencing the charge distribution along the BR-junction helix. Our results suggest that these bHLH proteins can bind DNA in particular conformational "states" that are determined by residues 5, 6, and 15, and that these "states" establish preferences for different CANNTG sites.

It is striking that the Twist-like DNA binding preference is associated with decreased specificity for particular CANNTG sites (Figures 8-10). This finding is consistent with the notion that these proteins bind DNA in a subtly different conformation than do those with MyoD- or E2A-like preferences. If recognition of the bound MyoD BR by a co-factor protein is important for activation of muscle-specific genes, our results imply that such a factor would recognize the particular BR conformation which is established by the A_5, T_6, and R_{15} residues. Alternatively, the association of a lack of function with decreased binding specificity is also consistent with the idea that these proteins might not be functional because they can recognize a wide variety of CANNTG sites in the genome. Thus, although these proteins could bind to *bona fide* muscle-specific regulatory regions, their effective concentration available to these sites would be decreased because of competition from the variety of other CANNTG sites to which they could bind.

Our studies of TIS11/TTP proteins have demonstrated that forced TTP expression can induce cultured cells to proliferate, then undergo apoptosis. This observation shows that TTP has broader effects on cellular regulatory pathways than is suggested directly from the analysis of TTP -/- mice. It is consistent with the notion that TTP is involved in numerous growth factor responses, and is linked to mechanisms that control cell proliferation. The synergism with which TTP and

TNFα induce apoptosis may be analogous to that observed between c-Myc and TNFα, and is consistent with potential involvement of TTP in TNFα responses. Our findings so far do not address directly how lack of TTP results in increased TNFα activity, but they indicate that the effects of TTP expression are likely to be complex. They also provide an assay with which we can analyze mutant forms of TTP or other family members, study TTP-interacting proteins, and search for potential TTP targets by assessing the effects of its expression on various cell cycle regulators.

By performing a two-hybrid screen and initiating biochemical experiments for identification of TTP-interacting factors, we will open up avenues for investigating how TTP functions at the molecular level. Such experiments will be required for gaining a mechanistic understanding of how TTP is involved in TNFα activity, and of how the three TTP/TIS11 proteins are involved in responses to various growth factors, including EGF.

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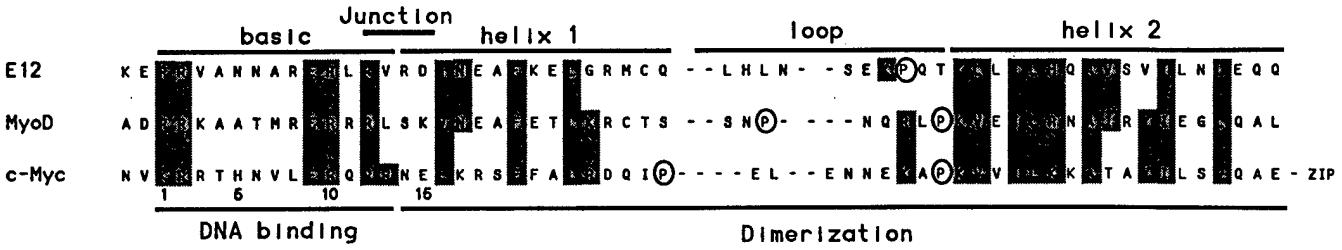
A.



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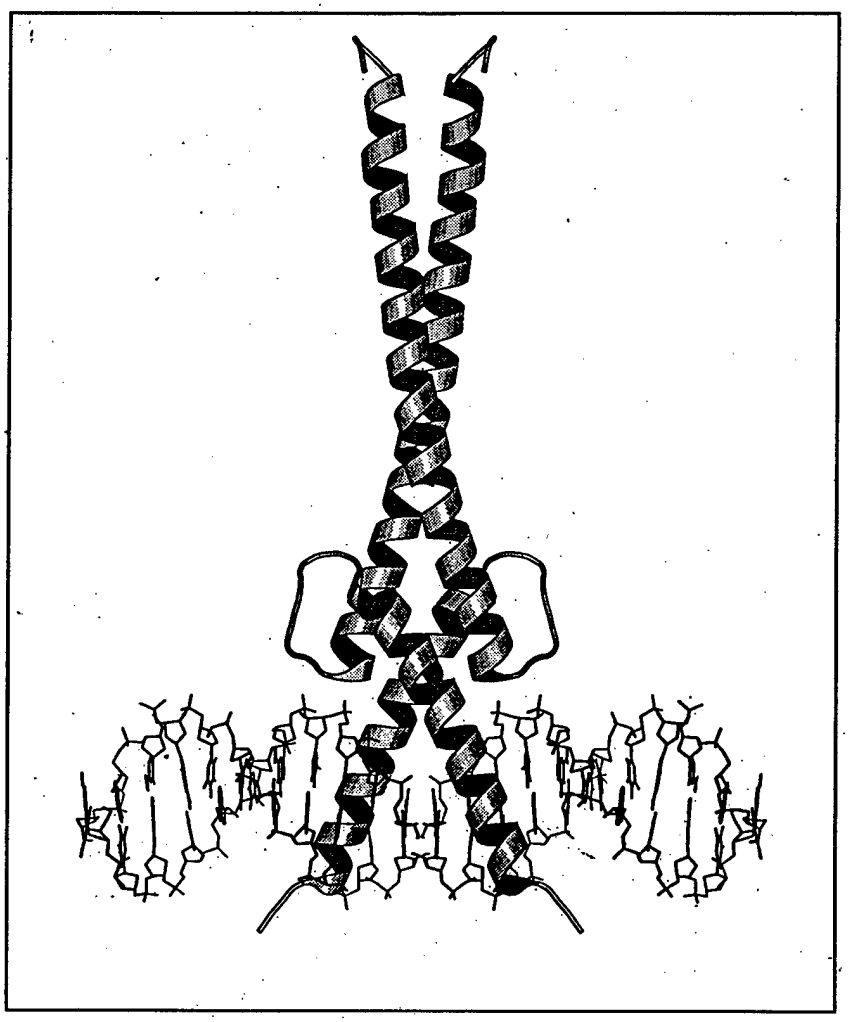


Figure 1. The bHLH domain. (A) The bHLH regions of human E12, MyoD, and c-Myc. Conserved residues are shaded, proline residues in the loop region are circled, and BR residues are numbered. ZIP indicates that the c-Myc HLH segment is followed immediated by a leucine zipper segment. (B) Ribbon diagram of the bHLH-ZIP protein Max, the dimerization partner for c-Myc (taken from Ferre d' Amare, 1993).

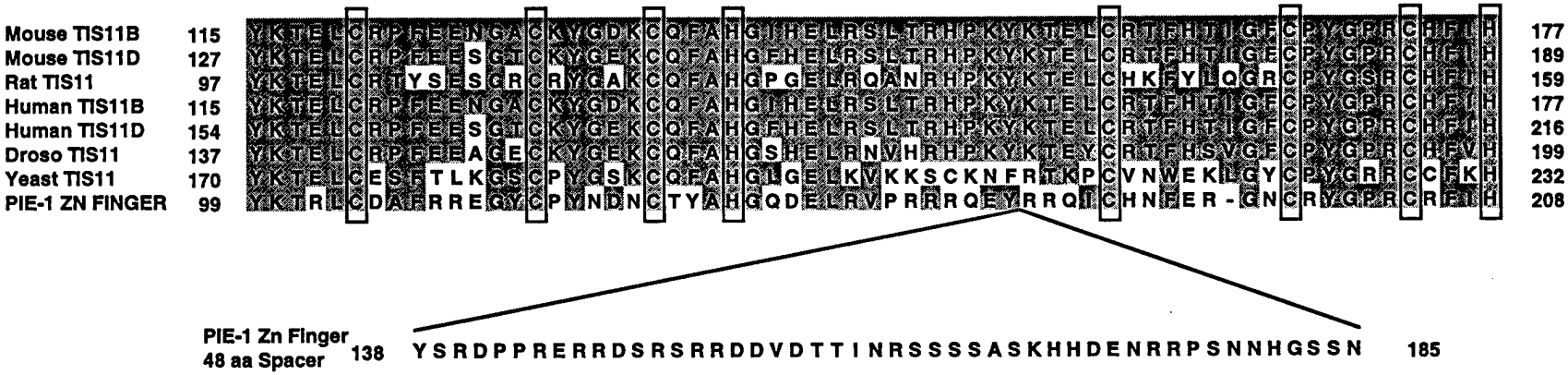


Figure 2. Alignment of representative TTP/TIS11 protein zinc fingers with those of C. elegans PIE-1. TIS11, TTP, and Nup475 are different names for the same protein. TIS11B and D are closely related to TTP/TIS11 within these zinc fingers, which are similar to those of the C. elegans PIE-1 protein. References are in the text.

Figure 3. Alignment of bHLH protein BRs, along with MyoD mutants. Mutant sequences, and their biological activities, are described in Davis and Weintraub (1992). Conserved residues are shaded, large subsituted regions are bracketed, and in the mutant BRs residues that correspond to MyoD are underlined. "Muscle" indicates induction of myogenesis in a tissue culture assay.

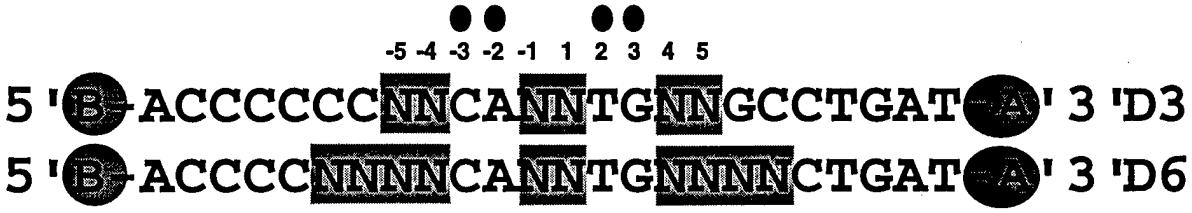


Figure 4. Random sequence libraries used for in vitro binding site selection (SAAB) experiments. Randomized bases are indicated by N, and primer sequences by A and B. These were derived from a muscle-specific regulatory sequence, and are described in Blackwell and Weintraub (1990), and Blackwell, et al. (1990).

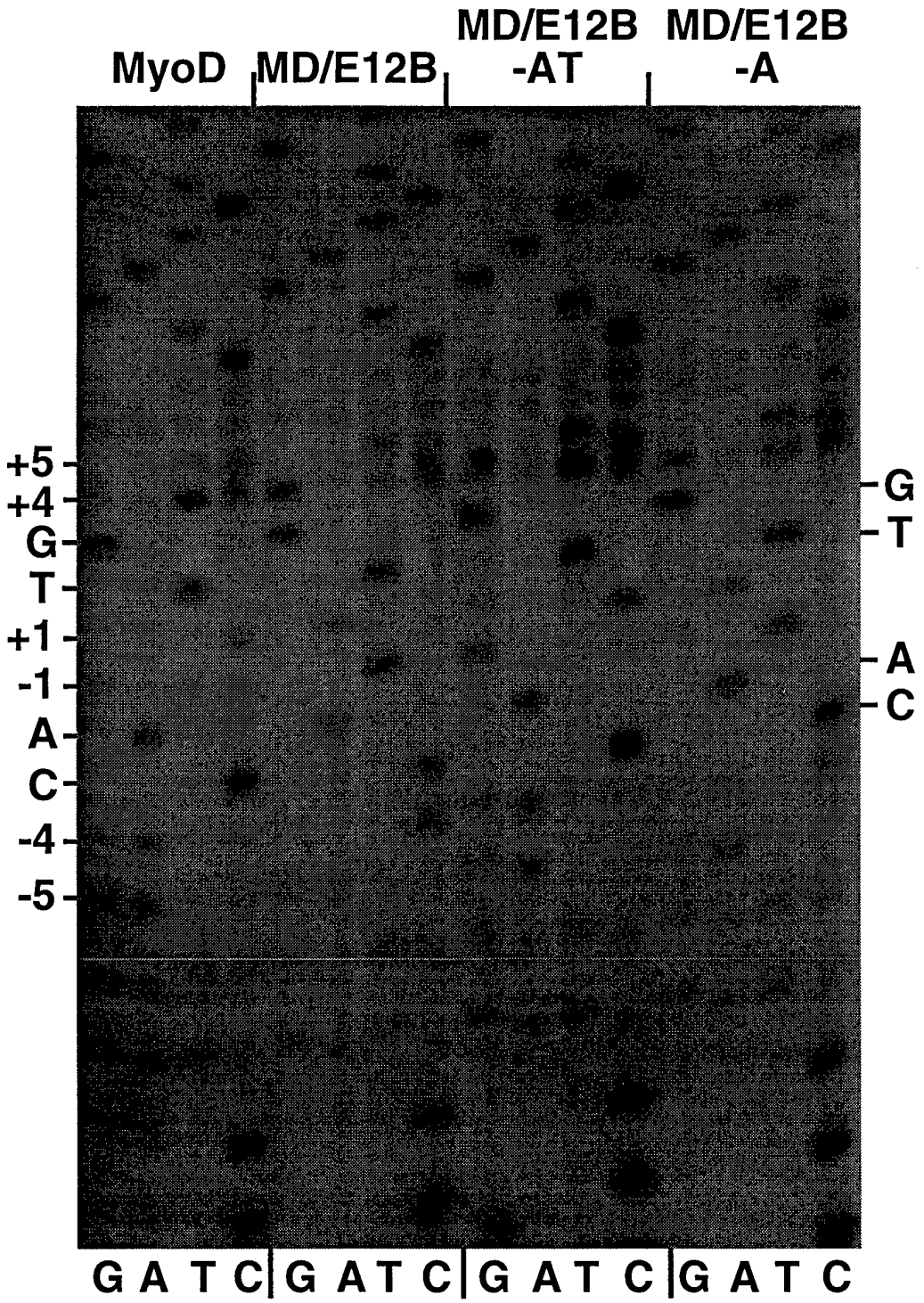


Figure 5. Binding site preferences of MyoD and the indicated BR mutants. These populations of prefered sltes were selected from the D6 random sequence library (Figure 4) for binding to the indicated purified proteins. The selected sites were sequenced as a pool as in Blackwell and Weintraub (1990).

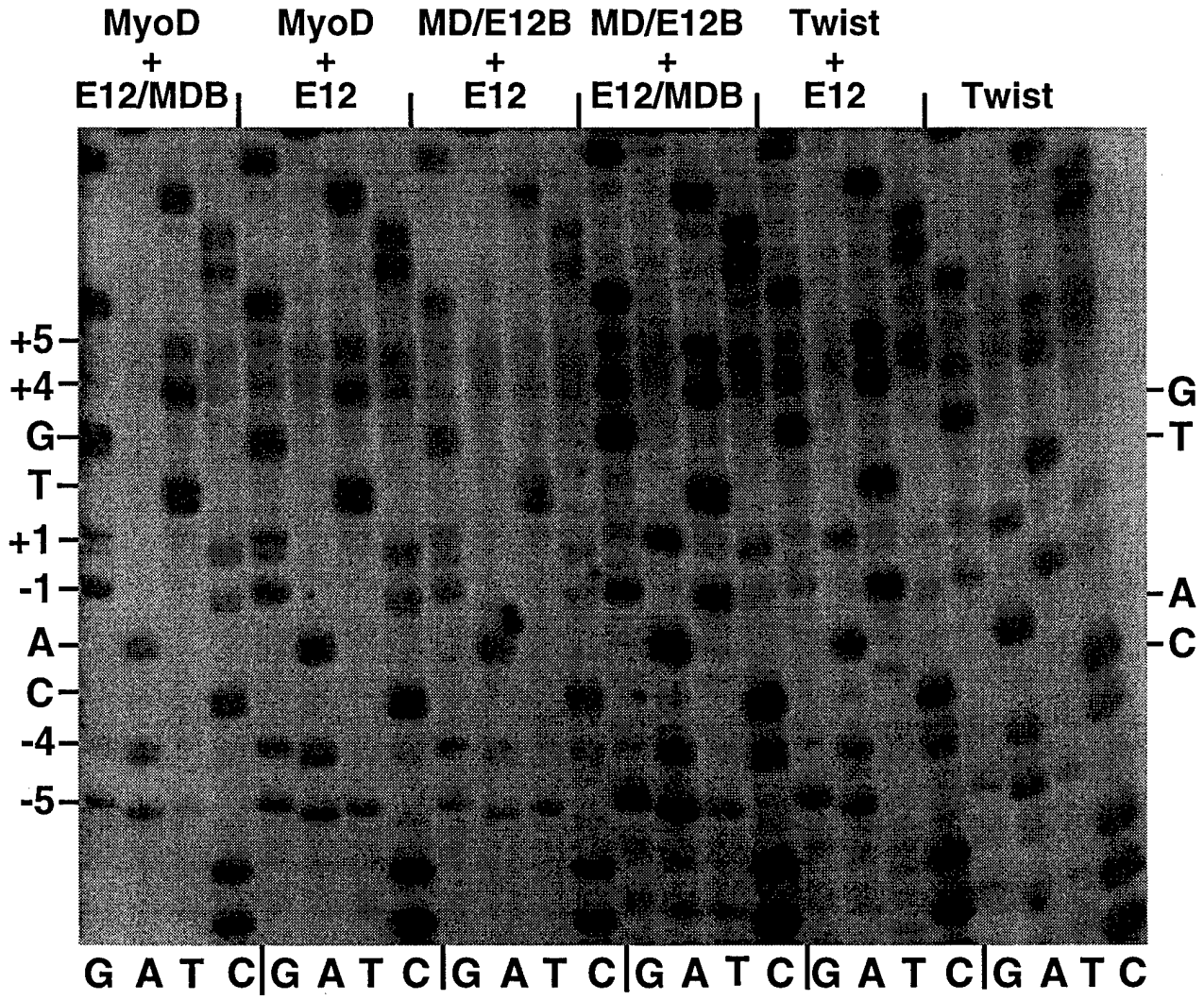


Figure 6. Binding site preferences of the indicated bHLH proteins. These populations of prefered sltes were selected from the D3 random sequence library (Figure 4) for binding to the indicated proteins, which were produced by in vitro translation. The selected sites were sequenced as a pool as in Blackwell and Weintraub (1990).

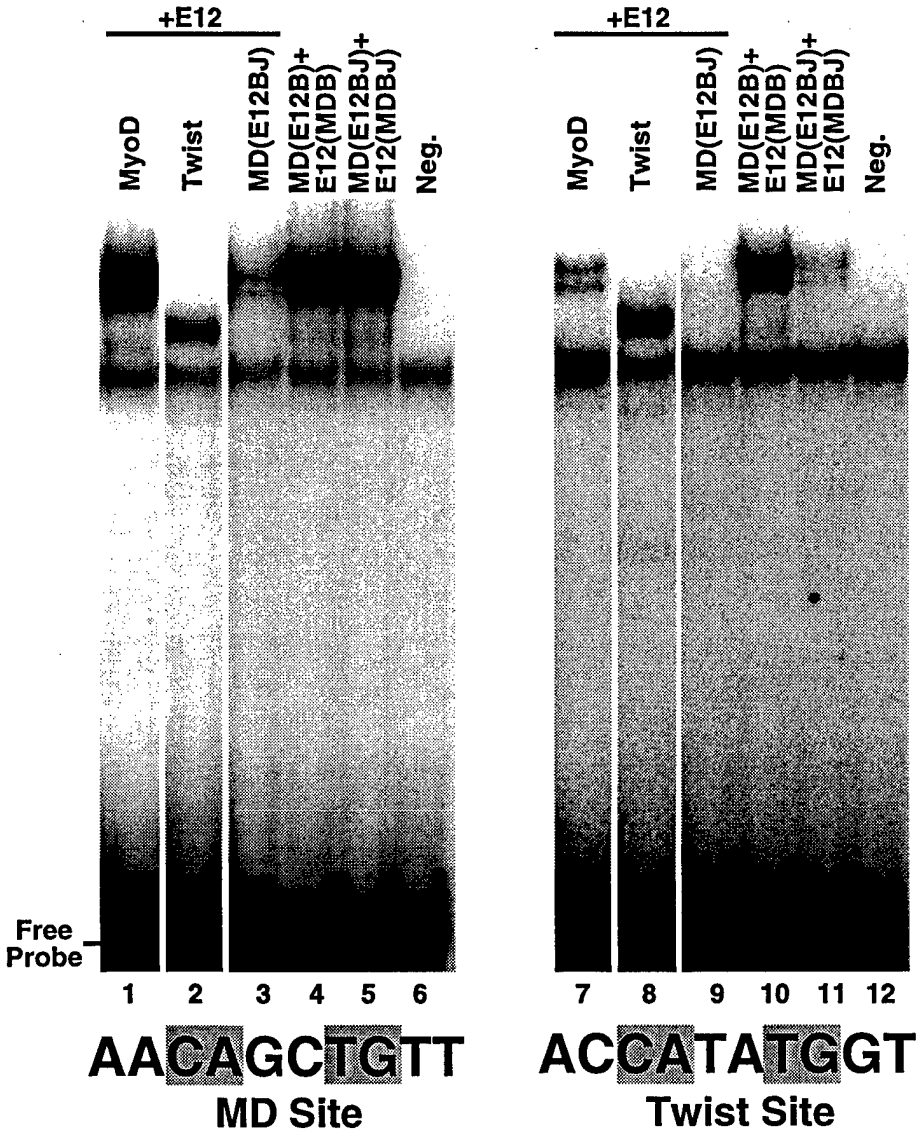


Figure 7. DNA binding specificity of E2A (E12) bHLH heterodimers. The indicated proteins were produced by in vitro translation, and assayed by EMSA for binding to sites preferred by MyoD and Twist.

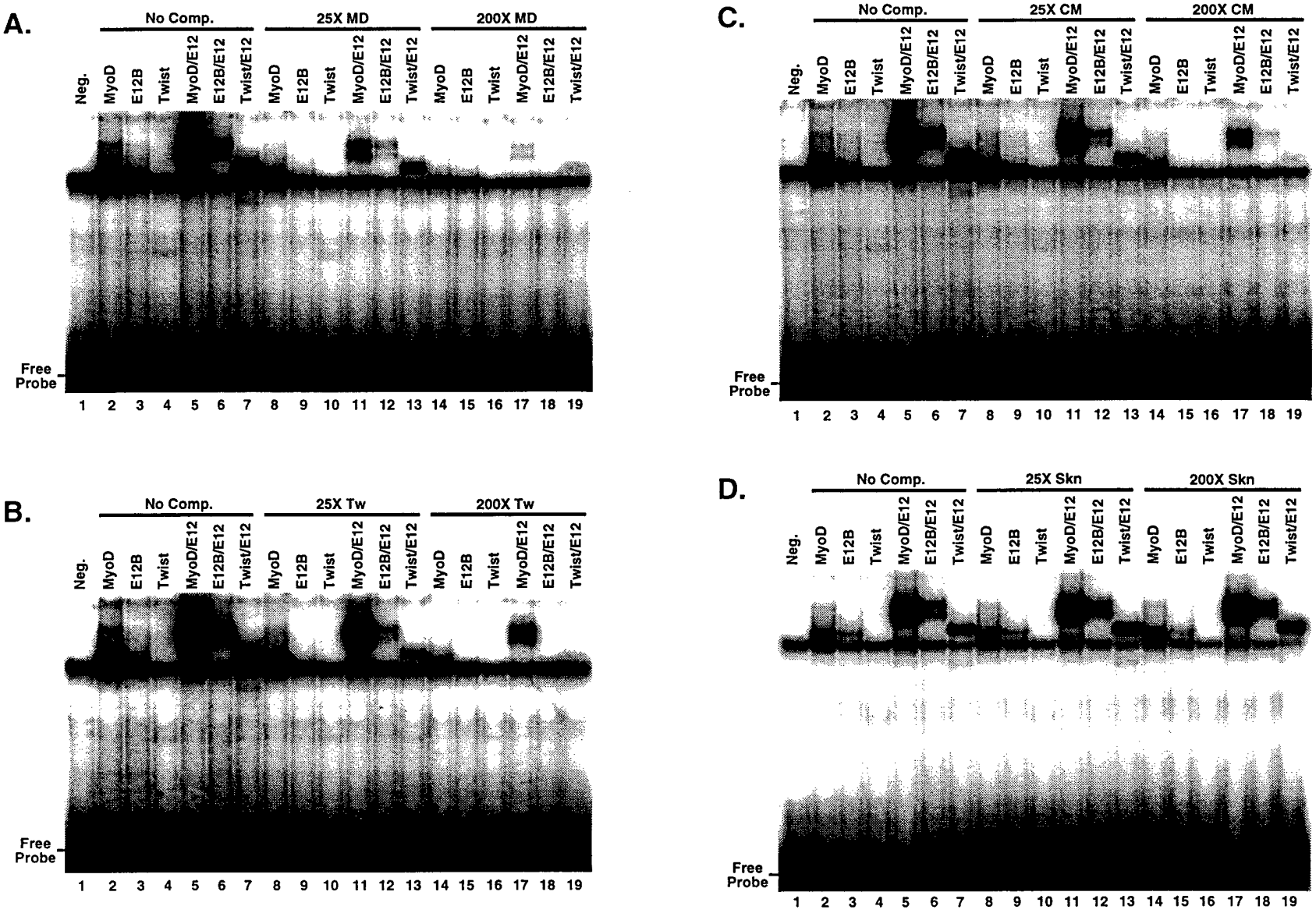
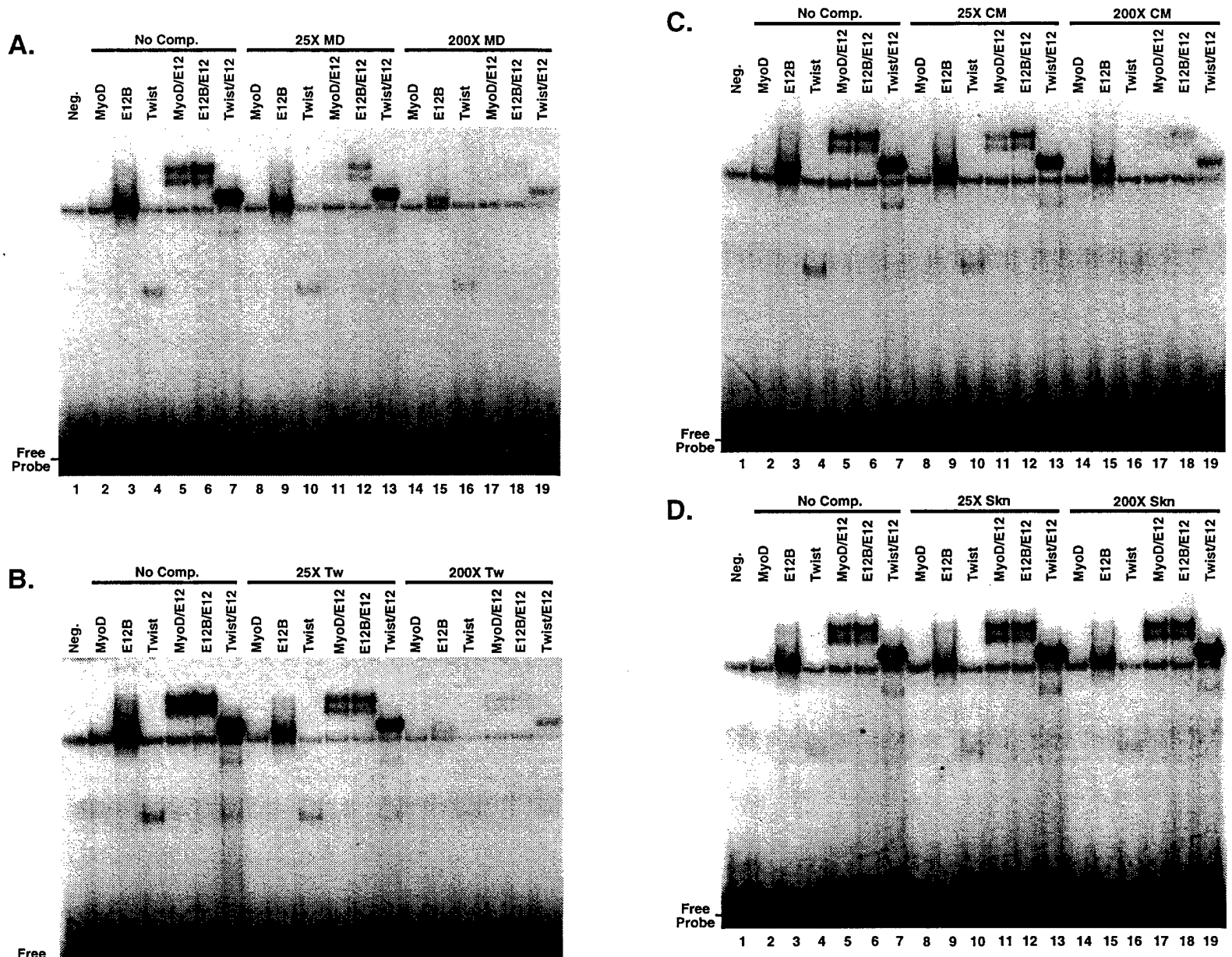
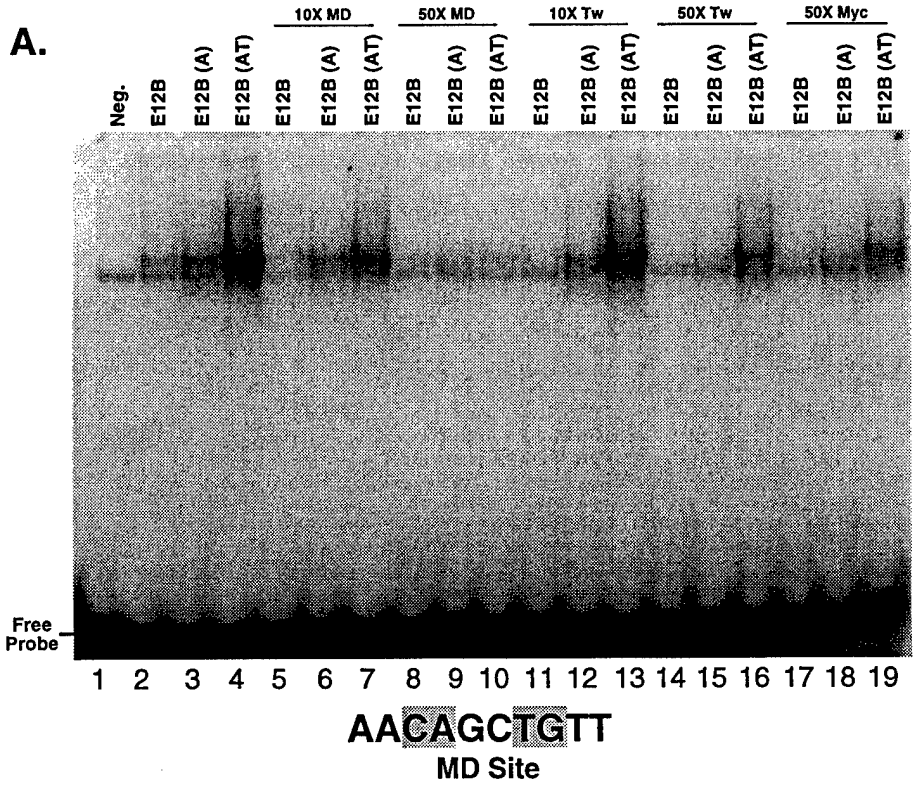


Figure 8. Specificity of bHLH protein binding to the prefered MyoD binding site, which is shown to the right. Proteins that were translated in vitro were assayed by EMSA for binding to the MyoD site, with binding competed by the MyoD (A), Twist (B), c-Myc (C), and Skn-1 (D) preferred sites. E12B refers to the MyoD/E12basic mutant. Proportions of competitors added relative to the labeled DNA are indicated above the gels.



ACCATATGGT
Tw Site

Figure 9. Specificity of bHLH protein binding to the prefered Twist binding site, which is shown to the right. Proteins that were translated in vitro were assayed by EMSA for binding to the Twist site, with binding competed by the MyoD (A), Twist (B), c-Myc (C), and Skn-1 (D) preferred sites. Proportions of competitors added relative to the labeled DNA are indicated above the gels.



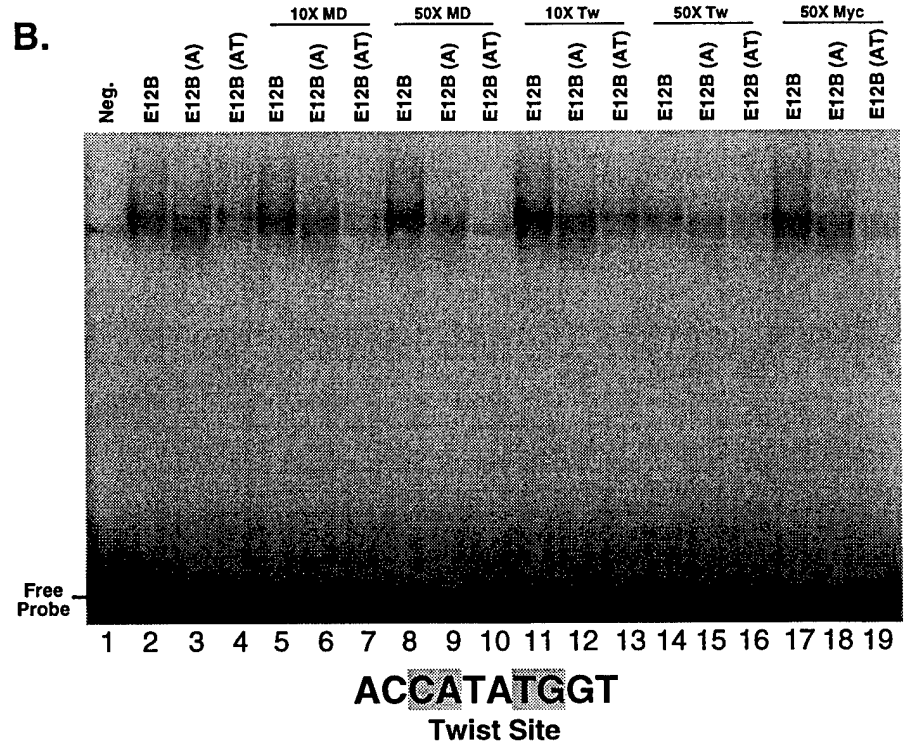


Figure 10. DNA binding specificity of MyoD BR mutants. The indicated proteins were produced by in vitro translation, and assayed by EMSA for binding to sites preferred by MyoD (A) and Twist (B). Competitor sites, and their concentrations relative to the labeled site, are indicated above each autoradiogram.

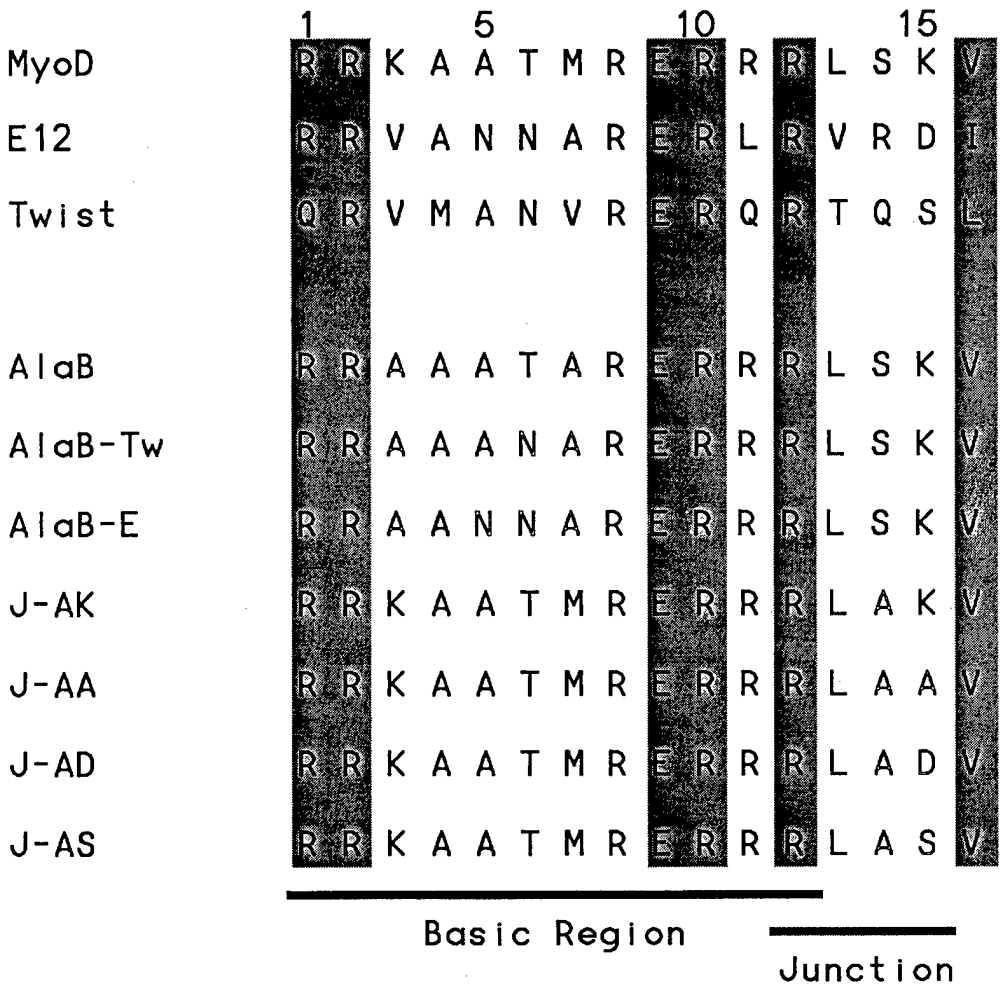


Figure 11. MyoD basic region mutants. Outlined letters indicate mutations.

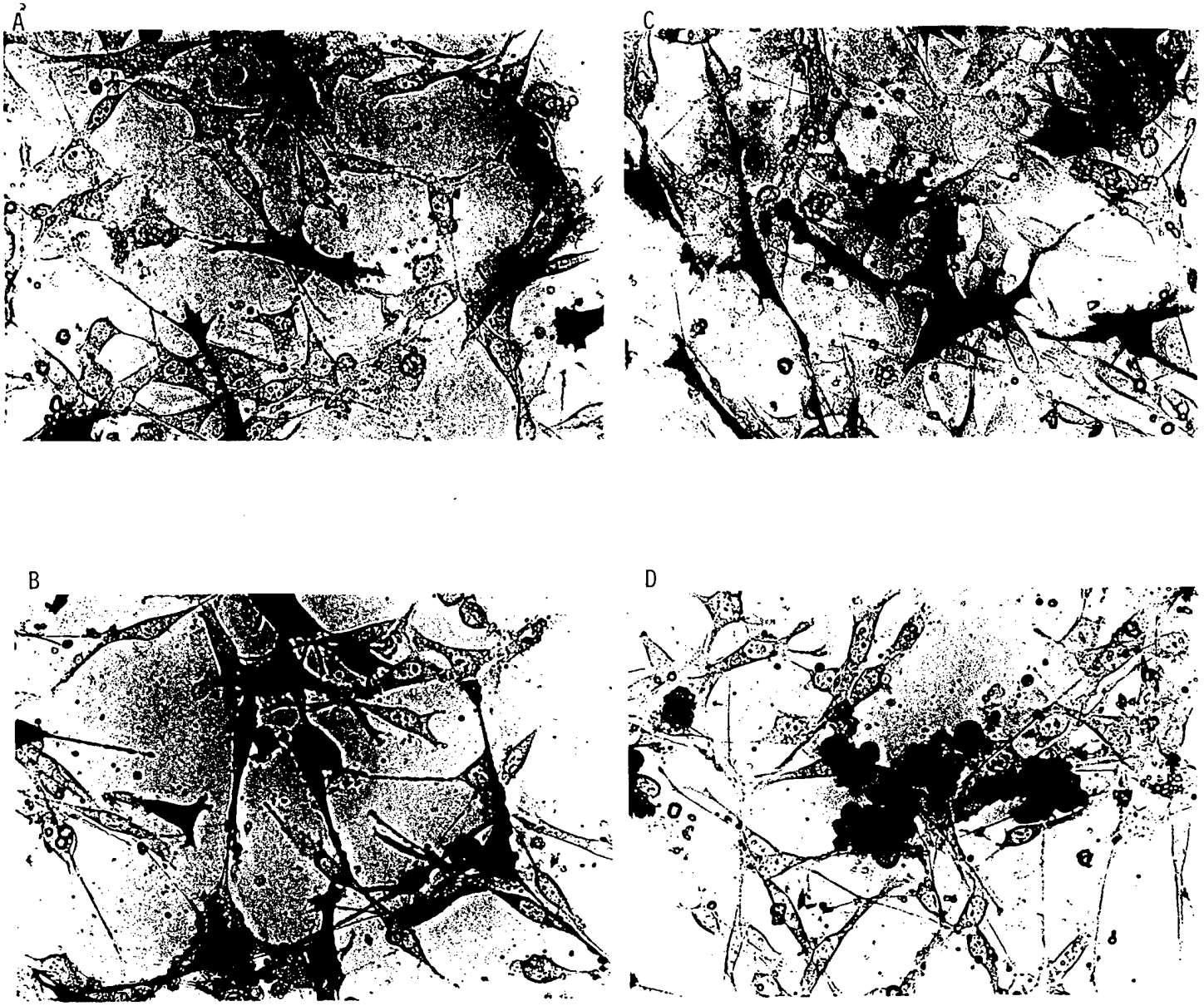


Figure 12. Expression of TIS11(TTP) in 3T3 cells. Cells were transfected with 1.5 μg of TTP expression vector, together with a β-galactosidase expression construct. TTP (B, D) and empty vector (A, C) transfections are shown at 24 hr. (A, B) and 48 hr. (C, D) after transfection.

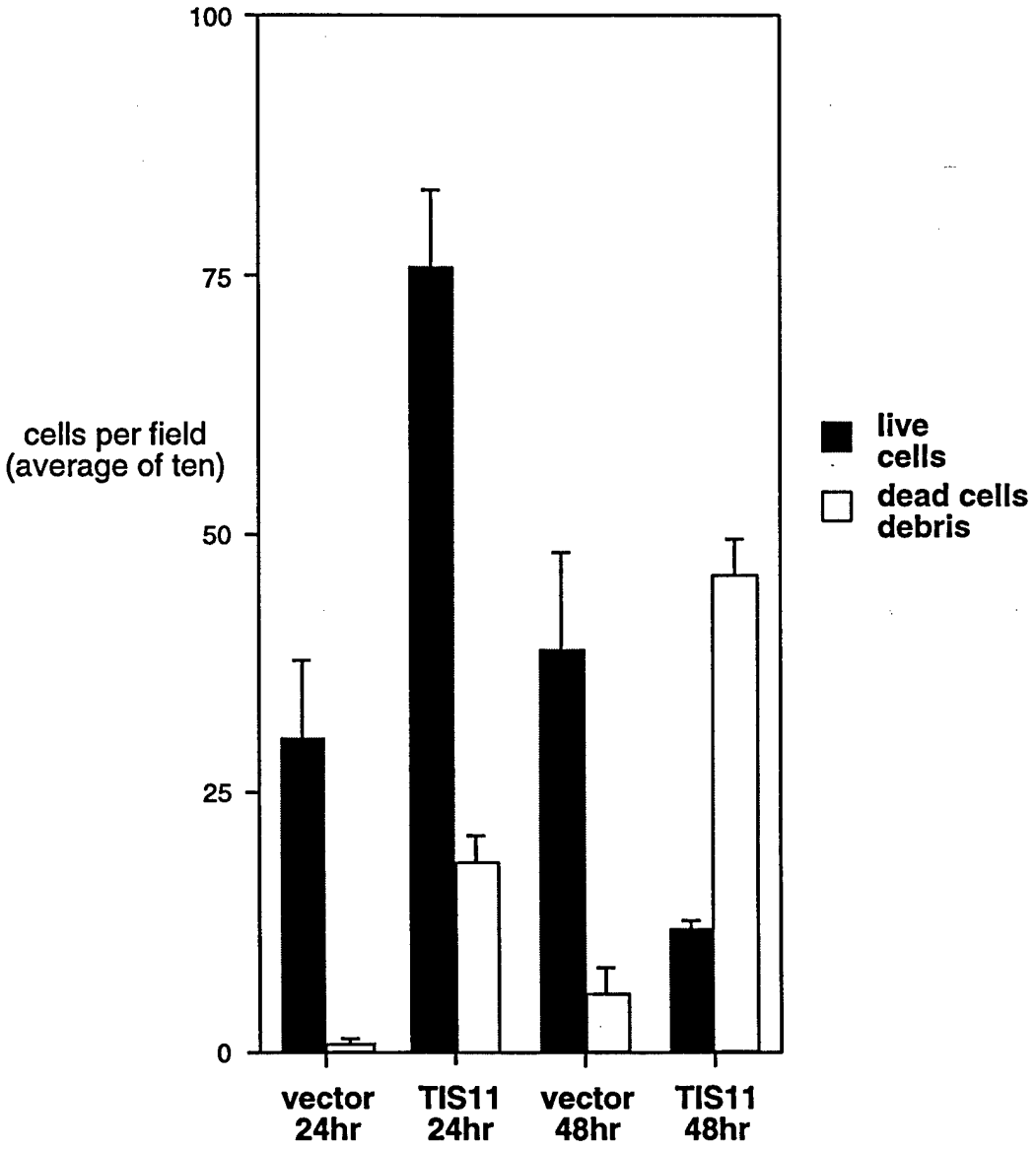


Figure 13. Forced expression of TIS11(TTP) induces proliferation and apoptosis. NIH 3T3 cells were transfected with the indicated expression construct, along with a CMV β-gal marker. Dead transfected cells were identified by their appearance, and only adherent cells were counted. Each experiment was performed in triplicate. Times indicated are post-transfection.

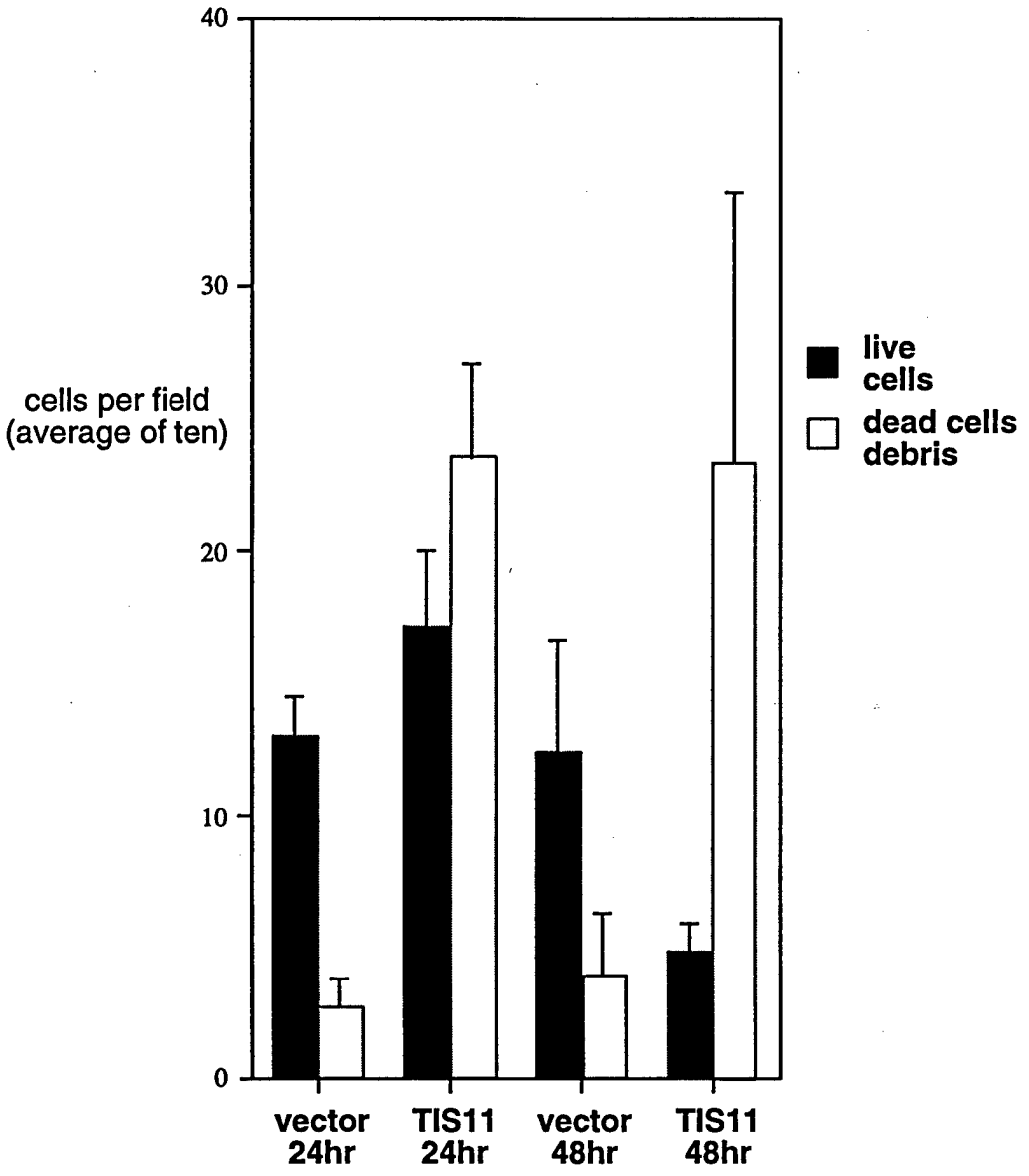


Figure 14. TIS11(TTP) and TNFα act synergistically to induce apoptosis. NIH 3T3 cells were transfected with the indicated expression construct, along with a CMV β-gal marker. Times indicated are post-transfection. TNFα (50 μg/ml) was added at 5 hr to all plates, then at 24 hr. fresh and medium and TNFα were added to the 48 hr. experiment. Dead transfected cells were identified by their appearance, and only adherent cells were counted. Each experiment was performed in triplicate. Note the decreased cell numbers, compared with the experiment in Fig. 12 which was done alongside.